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Immunogenicity of papaya mosaic virus-like particles fused to a hepatitis C virus epitope: Evidence for the critical function of multimerization

Jérôme Denis [a], Nathalie Majeau [a], Elizabeth Acosta-Ramirez [a,b], Christian Savard [a], Marie-Claude Bedard [a], Sabrina Simard [a], Katia Lecours [c], Marilène Bolduc [a], Christine Pare [a], Bernard Willems [d], Naglaa Shoukry [d], Philippe Tessier [a], Patrick Lacasse [e], Alain Lamarre [e], Réjean Lapointe [f], Constantino Lopez Macias [b], Denis Leclerc [a,*]

[a] *Centre de Recherche en Infectiologie, Pavillon CHUL, Université Laval, 2705 boul. Laurier, Québec, PQ, Canada*

[b] *Unidad de Investigación Médica en Inmunoquímica, 1er piso Hospital de Especialidades, Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social (IMSS), México D.F., Mexico*

[c] *Centre de Recherche sur la fonction, la structure et l'ingénierie des protéines CREPSIP, Department of Biochemistry and Microbiology, Laval University, Pavillon C-E Marchand, Québec, PQ, Canada*

[d] *Centre de recherche du CHUM, Hôpital St-Luc, Montréal, P.Q., Canada*

[e] *INRS-Institut Armand-Frappier, 531, boul. des Prairies, Laval, Québec, Canada*

[f] *Research Centre, Centre Hospitalier de l'Université de Montréal, Hôpital Notre Dame, Université de Montréal and Institut du Cancer de Montréal, Montréal, Québec, Canada*

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Abstract

Plant-virus-based vaccines have emerged as a promising avenue in vaccine development. This report describes the engineering of an innovative vaccine platform using the papaya mosaic virus (PapMV) capsid protein (CP) as a carrier protein and a C-terminal fused hepatitis C virus (HCV) E2 epitope as the immunogenic target. Two antigen organizations of the PapMV-based vaccines were tested: a virus-like-particle (VLP; PapMVCP-E2) and a monomeric form ($PapMVCP_{27-215}$-E2). While the two forms of the vaccine were both shown to be actively internalized *in vitro* in bone-marrow-derived antigen presenting cells (APCs), immunogenicity was demonstrated to be strongly dependent on antigen organization. Indeed, C3H/HeJ mice injected twice with the multimeric VLP vaccine showed a long-lasting humoral response (more than 120 days) against both the CP and the fused HCV E2 epitope. The antibody profile (production of IgG1, IgG2a, IgG2b, IgG3) suggests a Th1/Th2 response. Immunogenicity of the PapMV vaccine platform was not observed when the monomer PapMVCP-E2 was injected. These results demonstrate for the first time the potential of the PapMV vaccine platform and the critical function of multimerization in its immunogenicity.

Keywords: Vaccination; Virus-like particles (VLPs); Chimeric plant virus; Epitope carrier

Introduction

In general, vaccines against viral diseases are based on attenuated or chemically inactivated live viruses (Ada, 2001). In the case of vaccine development against new emerging infectious diseases such as human immunodeficiency virus (HIV), the application of this traditional approach raises obvious safety issues due to possible reversion, recombination or mutation. Therefore, the development of novel vaccine platforms is urgently needed and is considered a priority for global health improvement over the next 5–10 years, most notably in developing countries (Daar et al., 2002).

Among the numerous new approaches to vaccine development, virus-like particles (VLPs), made of viral nucleocapsids, have emerged as a promising strategy. VLPs are composed of viral structural proteins that retain the ability to self-assemble

* Corresponding author.
E-mail address: denis.leclerc@crchul.ulaval.ca (D. Leclerc).

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without requiring the presence of the viral genome: they have been shown to be highly immunogenic and avoid the above-mentioned safety issues (Noad and Roy, 2003). The ability of VLPs to stimulate an adaptative immune response relies on several properties: the average diameter of VLPs (<0.05 μm), which is optimal for uptake of the VLPs by dendritic cells (Fifis et al., 2004); efficient activation of the antigen presenting cells (APCs) (Lenz et al., 2003); induction of CD8+ activation by a cross-priming mechanism (Ruedl et al., 2002); potent stimulation of a B-cell mediated response by direct cross-linking of the BCR on B cells. This latter property was first demonstrated in studies using organic polymers decorated with haptens, which proved that 20–25 haptens spaced by 5–10 nm were enough for T-cell-independent B-cell activation (Dintzis et al., 1976), (Mond et al., 1995). These results were subsequently illustrated using various viral models, for example by comparing the immunogenicity of a multimeric antigen versus the immunogenicity of its less organized or soluble counterparts (Bachmann et al., 1995; Baschong et al., 2003; Dintzis et al., 1976; Justewicz et al., 1995; Loor, 1967; Milich and McLachlan, 1986) or by showing the ability of VLPs exhibiting a self-epitope to break tolerance (Bachmann et al., 1993; Chackerian et al., 2001; Jegerlehner et al., 2002). Thus, the displayed array of antigens in a repetitive structure on viral, or VLP, surfaces was proposed to be important for the B-cell mediated response. It was suggested that the high immunogenicity of hepatitis B virus (HBV) VLPs comes from its ability to synergize both T-cell-independent activation of B cells by direct cross-linking of BCR and T-cell-dependent activation of B cells (Milich and McLachlan, 1986).

To date, two VLP vaccines, HBV and human papillomavirus (HPV), have been shown to function efficiently in humans (Fagan et al., 1987; Harper et al., 2004). VLPs derived from other viral pathogens, such as HIV-1 and hepatitis C virus

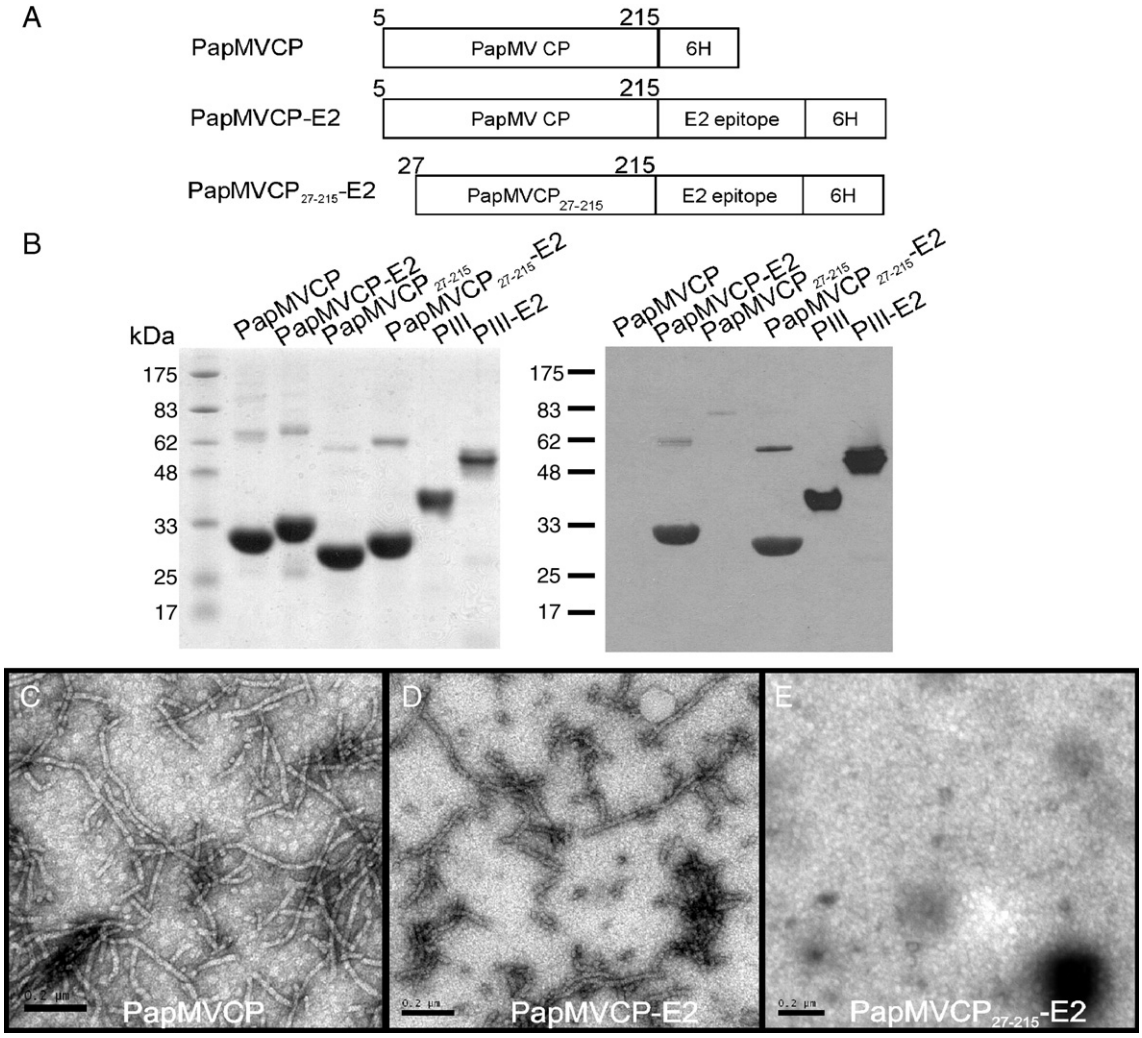

Fig. 1. Purification and characterization of recombinant proteins. (A) PapMVCP, the multimeric PapMVCP-E2 and the monomeric PapMVCP$_{27–215}$-E2 forms were fused to a 6xHis tag (6H) located at the C-terminus of the protein. The hepatitis C virus (HCV) peptide (E2$_{511–530}$) derived from envelope protein E2 is fused between the PapMVCP and the 6H tag in PapMVCP-E2 and PapMVCP$_{27–215}$-E2. (B) Purification of recombinant proteins by affinity chromatography using the 6H tag and a nickel column. The purity of the recombinant proteins was confirmed by SDS–PAGE (left), and the presence of the E2 epitope was confirmed by western blot using an anti-PIII-E2 antibody (right). (C) Electron microscopy of PapMVCP virus-like particles (VLPs), (D) PapMVCP-E2 VLPs and (E) PapMVCP$_{27–215}$-E2. Scale bars 200 nm.

(HCV), have also been generated, but their efficacy remains to be proven in humans (Noad and Roy, 2003). Alternatively, the use of VLPs from plant viruses as epitope presentation systems has triggered much interest. Indeed, plant viruses combine three characteristics essential to triggering a humoral response and memory: (1) they are comprised mainly of proteins that are highly immunogenic, (2) they are phylogenetically distant from the mammalian immune system, and (3) they possess a complex and repetitive organization. Cowpea mosaic virus (CPMV), tobacco mosaic virus (TMV), alfalfa mosaic virus (AlMV) and potato virus X (PVX) have been successfully produced in plants as vaccine platforms presenting peptides of interest (Canizares et al., 2005), and some vaccines have been proven to be protective in various diseases models (reviewed in Streatfield and Howard, 2003). Furthermore, the expression of Johnson grass mosaic virus coat protein (CP) in *E. coli* (Saini and Vrati, 2003) led to the formation of VLPs that were used as a recombinant vaccination platform conferring protection after challenge against Japanese encephalitis virus. Following the observation that expression of the papaya mosaic virus (PapMV) CP in *E. coli* also led to the self-assembly of VLPs composed of several hundred CP subunits organized in a repetitive manner (Tremblay et al., 2006), the potential of PapMV as a recombinant peptidic vaccine platform was tested. An epitope from the HCV E2 glycoprotein was fused to the PapMV CP, resulting in the production of a new recombinant VLP platform (PapMVCP-E2). The fused HCV peptide (511–530) belongs to a larger region (411–613) of the E2 glycoprotein containing three main antibody (Ab) determinants that can prevent the binding of HCV to its CD81 receptor on human cells (Lechner et al., 1998). Furthermore, the critical function of multimerization in triggering a humoral response, already demonstrated in mammalian virus models, was evaluated for the first time using a plant virus model, by comparing the immunogenicity of a recombinant plant VLP and its monomeric counterpart. A monomeric mutant was generated by deletion of the 26 first amino acids of the CP ($PapMVCP_{27–215}$); this protein (referred to as $PapMVCP_{27–215}$-E2 in this report) was used to express the same epitope as the multimeric protein and its performance as a vaccine platform was compared *in vivo* with the PapMV VLP-based vaccine platform. Importantly, this monomeric form of PapMV CP, which is unable to self-assemble in *E. coli*, has previously been reported as having the same secondary structure as native CP (Lecours et al., 2005).

In this report, we show that the recombinant multimeric PapMV vaccine platform is able to trigger a strong and long-lasting immune response against a C-terminal fused epitope. The antibody response profile directed against the epitope suggests a balanced Th1/Th2 response. Notably, the same platform lost its immunogenic properties when injected as a monomeric protein, validating the finding that a repetitive organization is one of the key properties of molecules triggering an immune response in the mammalian immune system. This is the first demonstration of the critical function of a multimeric structure for a plant-virus-based platform.

Results

Production of recombinant multimeric and monomeric forms of PapMV CP

Expression of PapMVCP in *E. coli* generates VLPs that are similar in appearance to the wild-type virus purified from the leaves of infected papaya plants (Tremblay et al., 2006). To examine the ability of PapMVCP fused to an epitope from the HCV E2 glycoprotein to assemble into VLPs, recombinant PapMVCP, PapMVCP-E2 and the monomeric form $PapMVCP_{27–215}$-E2 (Fig. 1A) were expressed in *E. coli* and purified using an easy procedure (see Materials and methods). SDS–PAGE analysis revealed that there were no significant differences in the yield of the various recombinant proteins (Fig. 1B, left panel), and immunoblotting directed against the E2 epitope indicated that no trimming of the peptide had occurred (Fig 1B, right panel). Note that the apparent molecular weights of PapMVCP-derived proteins on SDS–PAGE are slightly higher than expected (Fig. 1B, left panel; observed 30–33 kDa vs predicted 23–26 kDa), as previously noted (Lecours et al., 2005; Tremblay et al., 2006). As expected, an immunoblot using antibody directed to the E2 peptide confirmed that both the monomeric ($PapMVCP_{27–215}$-E2) and the multimeric form (PapMVCP-E2) harbor the E2 fusion (Fig. 1B, right panel). Endotoxin levels were always below 0.005 EU/μg of protein. Electron microscopy (EM) confirmed that addition of the E2 peptide at the C-terminus of PapMVCP did not affect the ability of the protein to self-assemble into VLPs (Fig. 1D), although the particles were notably shorter than VLPs formed by recombinant PapMVCP (Fig. 1C). Confirming previous results (Lecours et al., 2005), the monomeric form $PapMVCP_{27–215}$-E2 was unable to form VLPs (Fig. 1E). PapMVCP-E2 VLPs were variable in length, with a size range of 201 ± 80 nm. A length of 201 nm would represent 560 copies of the CP presenting the E2 peptide in a repetitive fashion. The secondary structures of the purified proteins were determined using circular dichroism spectrophotometry and were shown to be very similar to those previously reported (data not shown) (Lecours et al., 2005).

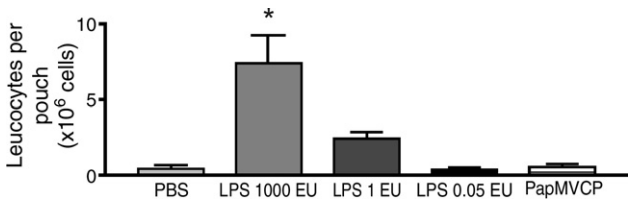

Fig. 2. Comparison of the pro-inflammatory response of PapMVCP and lipopolysaccharides (LPS). Dorsal air pouches were raised in 10- to 12-week-old CD1 mice. One milliliter of endotoxin-free PBS alone; PBS containing 0.05, 1 or 1000 EU/ml LPS; or recombinant PapMVCP (10 μg/ml) were injected into the air pouches. Migrating leukocytes in pouch exudates were harvested and counted 6 h after treatment. Data represent the mean ± SEM of 5 mice in each case. These results are representative of two identical and independent experiments. *Significant ($P < 0.05$) differences between cell recruitment levels as determined by Dunn's multiple comparison test.

Recombinant PapMV VLPs are weak inflammatory molecules

Some commonly used adjuvants, such as alum, have been described as mediating an inflammatory episode at the site of injection (Brewer, 2006). This characteristic can be very useful for the induction of a strong immune response. To test the pro-inflammatory properties of PapMVCP VLPs, we used the murine air pouch model. Injection of 10 μg of PapMVCP VLPs failed to induce the recruitment of leukocytes into the pouch of CD1 mice 6 h after treatment. In contrast, injection of lipopolysaccharides (LPS) at doses of 1000 and 1 EU was very effective in inducing the recruitment of leukocytes (Fig. 2). This result suggests that PapMVCP VLPs are not pro-inflammatory after 6 h and that the very low level of LPS in our proteins samples (<0.005 EU/μg) would not exert any notable immunogenic effects in subsequent experiments.

Comparative internalization of PapMVCP-E2 and PapMVCP$_{27–215}$-E2 in bone-marrow-derived dendritic cells

The uptake of VLPs by APCs was previously shown to be important for the induction of the immune response (Lenz et al., 2003; Gamvrellis et al., 2004). Therefore, we tested the capacity of the monomeric (PapMVCP$_{27–215}$-E2) and the multimeric

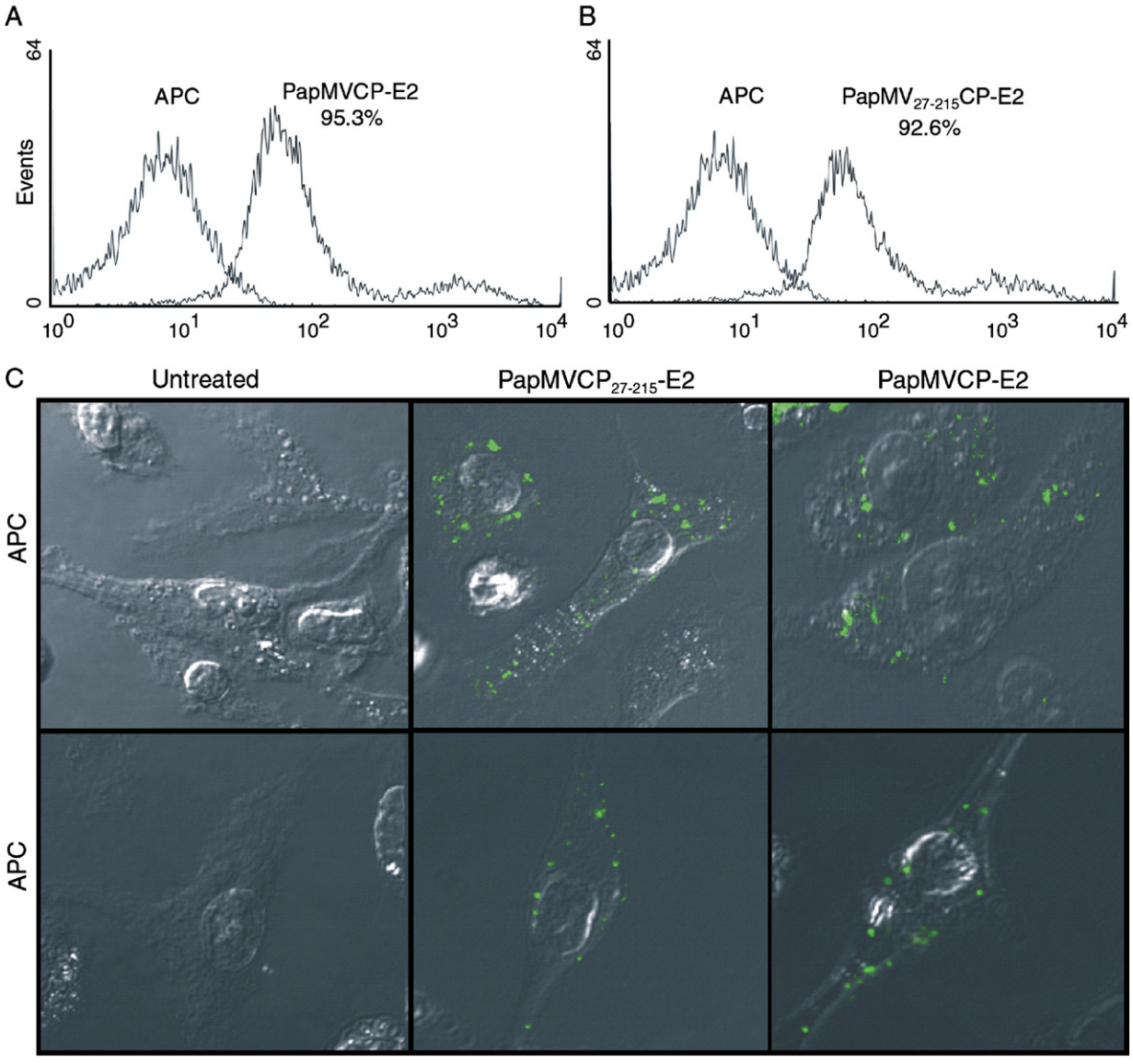

Fig. 3. Comparative internalization of PapMVCP-E2 and PapMVCP$_{27–215}$-E2 in antigen presentation cells (APCs) derived from bone marrow cells. 1×10^6 APCs were incubated for 2 h at 37 °C with 25 μg of PapMVCP-E2 or PapMVCP$_{27–215}$-E2. Cells were fixed and processed for immunofluorescence as described in Materials and methods. The recombinant proteins were detected with a mouse anti-rabbit antibody conjugated to alexa 488 (green), which in turn detected the primary PapMVCP rabbit polyclonal antibodies. (A, B) Internalization of PapMVCP-E2 VLPs (A) and PapMVCP$_{27–215}$-E2 (B) in APCs as measured by flow cytometry. (C) Internalization of the monomeric (PapMVCP$_{27–215}$-E2) or multimeric (PapMVCP-E2) PapMV vaccine platform in APCs analyzed by laser scanning confocal microscopy. Two representative images of each treatment are shown. Slides were scanned sequentially with a 488-nm laser line. Optical slices from the middle of the scanned cells are shown. An overlay of the phase contrast and the green channel is presented. The images are representative of two identical and independent experiments. The control (Untreated) is unstimulated cells labeled with PapMVCP rabbit polyclonal antibodies and the secondary alexa 488 anti-rabbit antibody.

(PapMVCP-E2) forms to be internalized in bone-marrow-derived APCs enriched in bone-marrow-derived dendritic cells (BMDDC). Equivalent amounts (25 μg) of PapMVCP-E2 and PapMVCP$_{27–215}$-E2 were incubated with 1×10^6 cells for 2 h at 37 °C. Flow cytometry analysis showed that APCs become efficiently immunolabeled (>90%) by both the multimeric (Fig. 3A) and the monomeric forms (Fig. 3B). To visualize the interaction between the recombinant proteins and the APCs, treated cells were observed by confocal microscopy. For both proteins, the immunolabeled PapMVCP signal was clearly vesicular, intracytoplasmic and perinuclear (Fig. 3B). Both recombinant proteins were efficiently internalized in APCs.

The humoral response depends on multimerization of the plant-virus-based vaccine platform

To examine the capacity of PapMVCP VLPs to induce an immune response, C3H/HeJ mice were injected subcutaneously with 25 μg of the recombinant VLPs (PapMVCP-E2) or 25 μg of the monomeric form (PapMVCP$_{27–215}$-E2). The amount of E2 peptide present in each dose is estimated at 2 μg. A booster dose was given on day 15 after primary immunization. Mice sera were assayed for anti-PapMVCP, PapMVCP$_{27–215}$ and anti-E2 peptide antibodies. Anti-CP IgG was clearly detected in mice immunized with PapMVCP-E2 on day 12, while only a low level of anti-CP was detected in the sera of mice vaccinated with PapMVCP$_{27–215}$-E2, even after the booster on day 15 (Fig. 4A). To detect anti-peptide antibodies, ELISA plates were coated with either the carrier protein alone (pIII) or carrier protein fused to the HCV E2 epitope (PIII-E2). The carrier protein fusion was used to detect anti-peptide IgG. The PIII carrier stabilizes the peptide and eases the purification because of its thermostability (Leclerc et al., 1998). Only sera of mice immunized with PapMV-E2 showed IgG antibodies against the carrier protein fused to the E2 peptide (PIII-E2), and the titer increased after the booster on day 15 (Fig. 4B). No antibody response was detectable against the carrier alone (PIII) for all sera (Fig. 4C). To examine the specificity of the antibody response elicited by the VLPs, sera from mice immunized with VLPs without the E2 peptide or with another peptide (derived from HCV E1 glycoprotein) were tested against PIII and PIII-E2. Antibodies to these latter proteins were not detected, confirming the specificity of the E2 antibodies (data not shown). The multimeric form was able to trigger a long-lasting response against both the CP and the E2 epitopes. Moreover, from day 23 to day 120, the antibody titer against the capsid decreased only on a 1 $\log_2$ scale (Fig. 4A), while the antibody titer against the peptide decreased on a 4 $\log_2$ scale (Fig. 4B). Isotyping of the anti-peptide antibodies revealed a Th1/Th2 balanced profile, with a prevalence of IgG1, IgG2a and IgG2b antibodies and a less pronounced production of IgG3 (Fig. 4D). A similar immunization protocol was performed in Balb/c mice with identical results: the same Th1/Th2 profile was observed with a similar IgG isotyping profile directed against both the PapMVCP and the E2 epitope (data not shown).

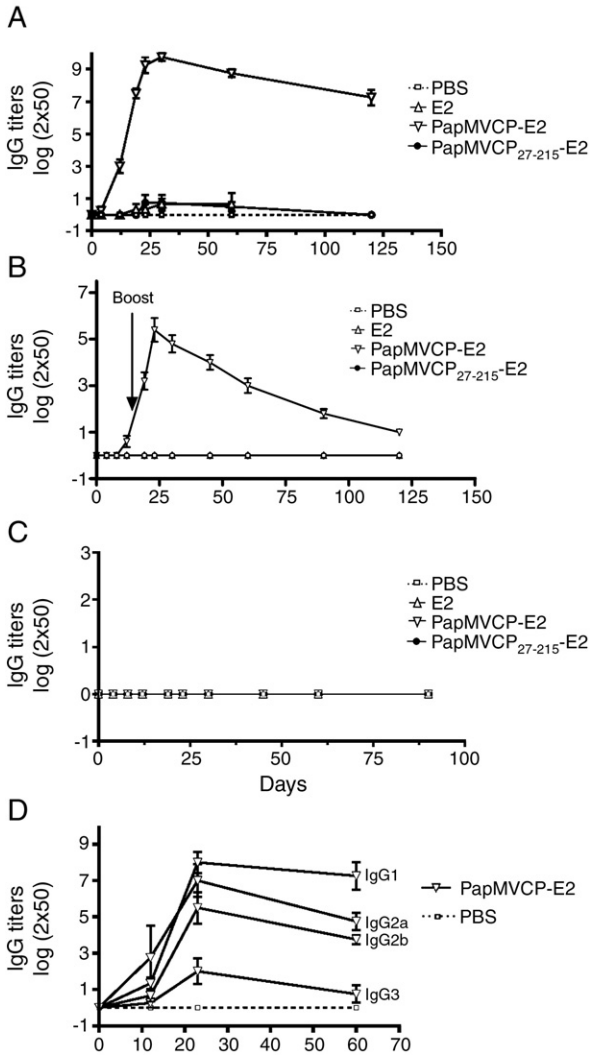

Fig. 4. Kinetics of the antibody response in C3H/HeJ mice injected subcutaneously twice (day 0 and day 15) with 25 μg of the multimeric PapMV vaccine platform (PapMVCP-E2), its monomeric counterpart (PapMVCP$_{27–215}$-E2), HCV E2 peptide (2 μg) alone or PBS. (A) IgG antibody response specific for the PapMV capsid protein. ELISA plates were coated with PapMVCP or PapMVCP$_{27–215}$. (B) IgG antibody response against the HCV E2 epitope. ELISA plates were coated with the HCV E2 peptide fused to the CaMV pIII carrier protein (see Materials and methods). (C) IgG antibody response to the CaMV pIII carrier protein alone. (D) IgG isotyping of the HCV E2 epitope antibody response, showing a balanced Th1/Th2 antibody isotyping profile. Only isotyping profiles of sera from mice injected with PapMVCP-E2 or PBS are presented in this figure. The results are expressed as antibody endpoint titer, defined as when the OD value is 3-fold higher than the background value obtained with a 1:50 dilution of serum from PBS-injected mice. Data represent the average of antibody titers from 4 (A) or 5 (B–D) mice. These results are representative of two identical and independent experiments. Black arrows on the graphs indicate the booster injections on day 15.

Reactivity of human sera against the HCV E2 peptide presented by the PapMV platform

An important issue in peptidic vaccination is good mimicry of the fused epitope. Consequently, to check if the peptide derived from the HCV surface glycoprotein E2 that is fused to the C-terminal of the PapMV CP can be recognized by serum from HCV-infected patients, sera from several healthy donors

Table 1
$E2^{511-530}$-specific antibody titres found in HCV-infected patients

Patient No/genotype	HD*	1/1a	2/1a	3/1a	4/1a	5/1a	6/1a	7/1a	8/3
PapMVCP VLPs	0	0	0	0	0	0	0	0	0
PapMVCP-E2 VLPs	0	0	0	0	0	0	1/800	0	0
pIII	0	0	0	0	0	0	0	0	0
pIII-E2	0	0	0	0	0	1/25	1/200	0	0

* HD: healthy donors, the sera of 15 healthy donors were mixed in equal proportion.

and eight HCV-infected patients were tested for reaction against PapMVCP-E2 VLPs (Table 1). Of the eight sera from HCV patients tested, one (patient no. 6) reacted with E2 peptide at the surface of PapMVCP-E2 VLPs, while the same serum did not react against PapMVCP VLPs (Table 1). When the E2 peptide was fused in the context of the carrier protein PIII, the same serum was reactive to the peptide. A second serum (patient no. 5) also reacted very weakly, close to the background level, to the E2 peptide. Although the same sera (patient no. 6) were clearly reactive with two anti-E2 detection systems (PIII/PIII-E2 and $PapMVCP_{27-215}$/$PapMVCP_{27-215}$-E2), the differences between the antibody titers could not be satisfactorily explained.

Discussion

This is the first report to compare the immunogenicity of multimeric and monomeric forms of a plant virus vaccine platform expressing the same B-cell epitope. Clearly, PapMV VLPs are immunogenic in mice whereas the monomeric forms are not. The VLP differs from the monomeric form in only two features: the multimerization of the subunit into a repetitive structure, and the RNA from *E. coli* located inside the VLPs. Both of these features were previously shown to play a role in the activation of the immune response; their combination in VLPs is likely to play an important role in their immunogenicity.

The central function of multimerization in triggering a humoral response in the PapMV vaccine platform model is consistent with previous studies in this area. Multimerization has been proven to be important in the immunogenicity of human viral vaccine platforms (Milich and McLachlan, 1986) or murine viruses (Bachmann et al., 1995), (Bachmann et al., 1993). Efficient direct cross-linking of BCR (Bachmann et al., 1995), (Zinkernagel, 2003) and facilitation of antigen internalization in APCs such as dendritic cells have been proposed as largely explaining the ability of VLPs to trigger an efficient antibody response and class-switching via cooperation of CD4+ activated lymphocytes (Gamvrellis et al., 2004). Any model designed to test the influence of antigen organization on humoral response has to address the two main phenomena influencing the level of activation of naive B cells, i.e., antigen binding to BCR and cross-linking of BCRs (Brunswick et al., 1988), separately. In other words, it is necessary to ensure that the lack of immunogenicity of a monomeric antigen compared to a multimeric antigen is not due to alteration of its binding capacity to specific BCRs rather than a reduced BCR cross-linking ability. Consequently, the tertiary structure required for BCR recognition must be similar for both the multimeric and monomeric forms of the antigen tested. As the monomeric form of PapMV CP was previously shown to have the same secondary structure as the multimeric CP, and was amenable to an extensive 3D NMR study (Lecours et al., 2005), our model allowed us to properly address the differential ability of the multimer and monomer to cross-link BCRs. This important structural control was not included in previous studies that generated monomeric antigen by denaturing VLPs by treating with SDS and 2-mercaptoethanol (Milich and McLachlan, 1986), treating flagellin with acid treatment (Feldmann and Easten, 1971) or by producing monomers in *E. coli* (Jegerlehner et al., 2002). None of these latter studies presented any evidence of secondary and tertiary structural conservation between the multimeric and monomeric forms. In our case, the monomeric form was unable to trigger a humoral response against the C-terminal presented peptide, while the multimeric form was able to induce a long-lasting response (120 days) against both the PapMVCP and the exposed epitope after two injections. This dramatic difference is unlikely to be due to degradation of the monomeric vaccine or trimming of the E2 fused peptide as our SDS–PAGE and immunoblotting analyses confirmed the integrity of the 2 vaccine forms and the uniform concentration of E2 peptides (Fig. 1). It is likely that the monomeric protein is unable to generate efficient activation of B cells, which could reasonably be attributed to a weak BCR cross-linking capacity. In contrast, injection of the multimer resulted in the creation of a pool of antibody-producing cells and memory B cells.

Since the class-switching (production of IgG1, IgG2a, IgG2b, IgG3) observed after injection of PapMVCP-E2 is a process that requires the contribution of CD4+ lymphocytes activated by APCs (Zinkernagel, 2003), internalization by bone-marrow-derived APCs was evaluated. As expected, the multimer was actively internalized by APCs. Interestingly, no difference in the magnitude of internalization into APCs was seen between the monomeric and the multimeric vaccine platform. In addition, differences between the monomeric and the multimeric vaccines in the *in vivo* capacity to activate DCs could not be demonstrated 6 h post-injection (data not shown). Therefore, the difference in immunogenicity could depend on the MHC class II processing of the antigen and subsequent activation of specific T cells, which, as previously demonstrated (Milich et al., 1997), may be more efficient with VLPs than with the monomeric form. The function of B cells as APCs could also be a key element; the same author also demonstrated that B cells could process HBV VLPs and activate a T-cell response 10^5 times more efficiently than DCs and MOs.

PapMVCP VLPs also contain an RNA of bacterial origin that was used as a scaffold for PapMV CP self-assembly in the bacteria (Tremblay et al., 2006). As in the purified plant virus, the RNA represents 5% (w/w) of the mass of the VLP (Erickson et al., 1976). In contrast, the monomeric form of the CP is not associated with RNA because its RNA binding activity is lacking (Lecours et al., 2005). Although the bacterial RNA is only a minor component of the VLP, we cannot exclude that it contributes to adjuvanticity by, for example, activating APCs as established previously (Kariko et al., 2005; Sugiyama et al.,

2005). Any potential contribution to the immunogenicity of PapMV VLPs of other bacterial components with strong adjuvanticity, such as LPS, was shown to be negligible in two different ways. Firstly, immunization with PapMV VLPs in LPS-sensitive mice (Balb/c, data not shown) and LPS hyporesponsive mice C3H/HeJ (Fig. 4) led to the same humoral immune response against both the platform and the C-fused epitope. Secondly, PapMV VLPs with very low LPS content (<0.005 EU/μg) were unable to trigger the recruitment of innate immune cells in the air pouch model after 6 h, contrasting with the high recruitment capacity of LPS (Fig. 2). Taken together, our results confirm the conclusion that the immunogenic effect of recombinant PapMVCP VLPs does not derive from LPS-like pro-inflammatory properties. The only other reported case of use of VLPs from a plant virus produced in *E. coli* made no mention of determination of the LPS content of the proteins injected or the influence of LPS content on the observed results (Saini and Vrati, 2003).

The Th1/Th2 profile of antibodies triggered by the PapMV recombinant protein without the use of any adjuvants suggests that this newly described vaccine platform potentially triggers a broader immune response than other plant-virus-based platforms. Indeed, antibody isotyping has previously suggested plant virus vaccine platforms to be biased toward a Th1 response (Marusic et al., 2001; McInerney et al., 1999) and cytokine secretion (Piazzolla et al., 2005). Even the presence of a commercial adjuvant with VLPs did not modify the Th1 bias (Marusic et al., 2001). A balanced Th1/Th2 antibody profile could increase the immune effector mechanisms of the humoral response such as neutralization or antibody-dependent cell-mediated cytotoxicity (ADCC) additional experiments would be necessary to investigate the potential of the PapMV platform to trigger these mechanisms in the HCV model. Moreover, the findings that the PapMV vaccine platform was able to trigger a pool of all antibody isotypes against the HCV E2 peptide and that serum from one HCV patient was clearly able to recognize the E2 peptide fused to the PapMV platform could indicate that this platform is suitable for triggering, for example, ADCC directed against hepatocytes exhibiting E2 protein on their surface as described in a clinical report by Nattermann et al. (2005), potentially helping to reduce the severity of the disease. In addition, a recent report (Leclerc et al., in press) demonstrated the capacity of the PapMV platform to activate human specific CD8+ lymphocytes *in vitro* by cross-priming. Taken together, these results suggest that the development of a vaccination platform based on PapMV VLPs against infectious diseases such as HCV, which requires both a protective CD8+ and a humoral response (Houghton and Abrignani, 2005), is a promising avenue of research.

Materials and methods

Cloning and engineering of the PapMV coat protein

The PapMV CP gene was amplified by RT/PCR from isolated viral RNA using primers 5′-AGTCCCATGGCATCCACACCCAACATAGCCTTC-3′and 5′-GATCGGATCCTTACTAATGGTGATGGTGATGGTGACGCGTGGTACTAGTTTCGGGGGGTGGAAGGAATTGGATGGTTGG-3′ and cloned as an *Nco*I/*Bam*HI fragment in pET 3D (New England Biolabs). To generate the PapMVCP-E2 construct, CCACCGATCGTAGCGGTGCGCCGACCTACAGCTGGGGTGCGAACGATACGCGTCATG-3′ and 5′-CATGACGCGTATCGTTCGCACCCCAGCTGTAGGTCGGCGCACCGCTACGATCGGTGGTACCCACCACCACACTAGTGATC-3′ were annealed together and digested with *Spe*I and *Mlu*I before ligation into the *Spe*I/*Mlu*I-linearized PapMVCP clone. The expression vector for PapMVCP$_{27–215}$-E2 was constructed from the PapMVCP-E2 plasmid as follows: two oligonucleotides (including an *Nco*I restriction site) designed to delete the 26 first amino acids of the PapMV CP were used for PCR: forward 5′-AGTCCCATGGCCGATCCAACGTCCAATCTTCTG-3′ and reverse 5′-ACGTCCATGGTATATCTCCTTCTTAAAG-3′. The PCR product was then self-ligated. The expression vector for PapMVCP$_{27–215}$ was derived from the PapMVCP plasmid following the same procedure as for the construction of the PapMVCP$_{27–215}$-E2 clone. The sequences of all PapMV clones were confirmed by DNA sequencing.

Cloning and engineering of the carrier protein (PIII)

A plasmid designed to express the cauliflower mosaic virus (CaMV) pIII protein in *E. coli* has been previously described (Leclerc et al., 1998). A truncated version of CaMV pIII comprising the 74 N-terminal amino acids was generated using primers 5′-AAACCCGGGGAATTCACCATGGCTAATCTTAATCAGATCCAAAAG 3′ and 5′-GATCGGATCCTAACGCGTGGTACTAGTAGGTTGGGTACCTAAGGCTTC-3′. This truncated version harbored unique *Spe*I and *Mlu*I sites that are convenient for cloning a peptide in fusion with pIII. To generate a PIII-E2 construct, the oligonucleotides E2 5′-GATCACTAGTGTGGTGGTGGGTACCACCGATCGTAGCGGTGCGCCGACCTACAGCTGGGGTGCGAACGATACGCGTCATG-3′ and 5′-CATGACGCGTATCGTTCGCACCCCAGCTGTAGGTCGGCGCACCGCTACGATCGGTGGTACCCACCACCACACTAGTGATC-3′ were annealed together and digested with *Spe*I and *Mlu*I before ligation into the PIII clone linearized with *Spe*I and *Mlu*I. The sequences of PIII clones were confirmed by DNA sequencing. The *E. coli* expression strain BL21(DE3) RIL (Stratagene) was transformed with PIII constructs, and protein expression was induced with 1 mM IPTG for 16 h at 25 °C. Cells were pelleted by centrifugation and resuspended in Tris–HCl pH 7.0, 20 mM NaCl, 1 mM EDTA and 0.02 mM PMSF before lysis by sonication. The protein extracts were then heated at 65 °C for 15 min and centrifuged at 15,000×*g* for 20 min at 4 °C. Incubation at 65 °C was repeated a second time. The samples were incubated at 80 °C for 15 min and centrifuged again for 20 min at 15,000×*g*. The chimeric pIII protein is resistant to heat and remains soluble in the supernatant after this treatment. The NaCl concentration was adjusted to 150 mM and protein extracts were separated on a Sephadex G-50 column to achieve 95% purity. The PIII protein is referred to as the carrier protein in the text.

Expression and purification of PapMVCP, PapMVCP-E2, PapMVCP$_{27–215}$ and PapMVCP$_{27–215}$-E2

Expression and purification of PapMVCP constructs were performed as previously described with minor modifications (Tremblay et al., 2006). Briefly, the bacteria were lysed through a French press and then loaded onto a Ni^{2+} column, washed with 10 mM Tris–HCl/50 mM Imidazole/0.5% Triton X100 (pH 8), then with 10 mM Tris–HCl/50 mM Imidazole/1% Zwittergent (pH 8) to remove endotoxin contamination. For the PapMVCP and PapMVCP-E2 proteins, the eluate was subjected to high speed centrifugation (100,000×*g*) for 120 min in a Beckman 50.2 TI rotor. The VLP pellet was resuspended in endotoxin-free PBS (Sigma). Following elution of the PapMVCP$_{27–215}$ and PapMVCP$_{27–215}$-E2 proteins, the solutions were dialyzed against PBS using a 6–8 kDa molecular weight cut-off membrane (Spectra). The E2 peptide was synthesized by GLBiochem (Shanghai) Ltd and resuspended in endotoxin-free PBS (Sigma). Protein solutions were filtered using 0.45 μM filters before use. The purity of the proteins was determined by SDS–PAGE and confirmed by western immunoblot analysis using mouse polyclonal antibodies generated against PIII-E2. The amount of protein was evaluated using a BCA protein kit (Pierce). The level of LPS in the purified protein was evaluated with the Limulus test according to the manufacturer's instructions (Cambrex) and was below 0.005 endotoxin units (EU)/μg of protein.

Electron microscopy

Proteins were diluted in PBS and were absorbed for 3 min on carbon-coated formvar grids. The grids were washed twice with deionized water and stained with 2% uranyl acetate for 10 min at room temperature. The grids were then observed on a Jeol JEM220FS transmission electron microscope. Average VLP length was evaluated by measuring 100 VLPs using Adobe Photoshop software.

Immunization

Five 4-to 8-week-old C3H/HeJ mice (Charles Rivers Laboratories) were injected subcutaneously with 25 μg of PapMVCP-E2, PapMVCP$_{27–215}$-E2 or the equivalent amount of the E2 peptide (2 μg) or endotoxin-free PBS (Sigma). Primary immunization was followed by one booster dose given 2 weeks later. Blood samples were obtained at different time points and stored at −20 °C until analysis. All the experimental protocols were approved by the Laval University animal protection committee.

ELISA quantification

Costar High Binding 96-well plates (Corning, NY, USA) were coated overnight at 4 °C with 100 μl/well of P3, P3E2, PapMVCP, PapMVCP$_{27–215}$, or PapMVCP-E2 diluted to a concentration of 1 μg/ml in 0.1 M $NaHCO_3$ buffer pH 9.6. The plates were blocked with PBS/0.1% Tween-20/2% BSA (150 μl/well) for 1 h at 37 °C. After washing three times with PBS/0.1% Tween-20, sera were added in 2-fold serial dilution (beginning with 1:50) and incubated for 1 h at 37 °C. Following incubation, the plates were washed three times and incubated with 100 μl of peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b (all from Jackson Immunoresarch) or IgG3 (Rockland) at a dilution of 1:10,000 in PBS/0.1% Tween-20/2% BSA for 1 h at 37 °C. After three washes, the presence of IgG was detected with 100 μl of TMB-S according to the manufacturer's instructions; the reaction was stopped by adding 100 μl of 0.18 mM H_2SO_4 and the OD was read at 450 nm. The results are expressed as antibody endpoint titer, determined when the OD value is 3-fold the background value obtained with a 1:50 dilution of serum from PBS-injected mice. For the determination of antibody levels in human sera, the same conditions were applied, except that the peroxidase-conjugated goat anti-human IgG as secondary antibodies were used at a dilution of 1:80,000. Sera from infected HCV patients were provided by B. Willems (Hopital Saint Luc, CHUM): the results are expressed as antibody endpoint titer, defined as the point at which the OD value is 3-fold the background value obtained with a 1:25 dilution of serum from a pool of sera from 15 non-infected patients.

Air pouches in mice

Air pouches were raised in 10- to 12-week-old CD1 mice (Charles River Laboratories). Experimental protocols were approved by the Laval University animal protection committee. Air pouches were raised on the dorsum by subcutaneous injection of 3 ml of sterile air on days 0 and 3. On day 7, 1 ml of recombinant PapMVCP (1 to 10 μg/ml), LPS (10 μg/ml) or PBS was injected into the air pouches. Six hours after treatment, the mice were killed by asphyxiation using CO_2. The air pouches were washed once with 1 ml PBS–5 mM EDTA and then twice with 2 ml of PBS–5 mM EDTA, and the exudates were centrifuged at 500×*g* for 5 min at room temperature. Cells were counted with a hematocytometer following acetic blue staining.

Bone marrow cell extraction and differentiation of APCs

Bone marrow progenitors cells were obtained from the femurs of Balb/c mice and cultured for 6 days in dendritic cells differentiation bone marrow medium (95% RPMI with 1% penicillin–streptomycin and supplemented with 5% X63-GM-CSF supernatant media culture; the X63-GM-CSF cell line was provided by B. Ludewig, Research Department, Cantonal Hospital, St. Gallen, Switzerland). Medium was partially replaced on days 2 and 4. On day 6, the medium was replaced by medium without LX63-conditioned medium. On day 7, enrichment of APCs was verified by flow cytometry using FITC anti-CD11c and PE-Cy5.5 anti-CD11b surface markers (BD Biosciences). The preparation contained 25% of CD11c+ Cd11b+ cells, and more than 80% of CD11b+ cells. We refer to this preparation as APCs.

Flow cytometry

To evaluate internalization of the PapMVCP-E2 or PapMVCP$_{27–215}$-E2 in APCs, 1 million bone-marrow-derived APCs were incubated for 2 h at 37 °C with either 25 μg of PapMV-E2 or PapMVCP$_{27–215}$-E2. Briefly, cells were blocked with PBS containing 10% FBS and anti-CD16/CD32 (1 μg/1 million cells) for 15 min at 4 °C. After 2 washes with PBS, cells were fixed with PBS/2% paraformaldehyde for 10 min at room temperature. After 2 washes with permeabilization buffer (PBS 10%/FBS 0.2%/Triton X-100), cells were incubated for 45 min at 4 °C with rabbit polyclonal antibodies diluted 1:200 in permeabilization buffer. After 2 washes with permeabilization buffer, cells were incubated for 45 min at 4 °C with the secondary antibodies (anti-rabbit IgG alexa 488; Molecular Probes) diluted 1:5000 in permeabilization buffer. After washing with PBS, cells were immediately analyzed with an EPICS-XL cytofluorometer. Data analysis was performed using WINMDI2.8. The rabbit polyclonal Ab used for detection was produced in our own facilities: rabbit preimmune serum was used as a negative control.

Confocal microscopy

APCs were grown (200,000 cells/well) in 12-well plates (Corning, NY, USA) containing sterile slides in the bottom following the differentiation protocol described above. For antigen internalization studies, 5 μg of antigen/200,000 cells was used. The fixation, permeabilization and primary and secondary antibodies incubation steps were as described for flow cytometry. Slides were analyzed immediately with a Fluoview Fv300 confocal microscope fitted with a ×60 oil immersion objective. Fluorescence images were acquired sequentially to avoid non-specific channel interference and by *x–z* sectioning. Pictures were then digitally processed with Image J software. Optical slices from the middle of the scanned cells are shown in the figures.

Statistical analysis

Nonparametric Kruskal–Wallis and Dunn's multiple comparison tests were used for statistical analysis. A value of $P<0.05$ was considered statistically significant. Statistical analyses were performed with the program PRISM 3.03.

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